Docket No.: AREX-P02-004
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:	Madiyalakan *et al.*	Confirmation No.:	4505
Application No.:	09/152,698	Art Unit:	1643
Filed:	September 2, 1998		
For:	THERAPEUTIC COMPOSITIONS THAT PRODUCE AN IMMUNE RESPONSE	Examiner:	K. A. Canella

DECLARATION UNDER 37 C.F.R. § 1.132 OF BIRGIT SCHULTES

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Birgit C. Schultes, Ph.D., of 12 Monadnock Road, Arlington, MA, hereby declare and state as follows:

1. I am Senior Director of Research at Unither Pharmaceuticals. I am also an inventor of the subject application. I have been conducting research in the field of tumor immunology for approximately 16 years. A copy of my *curriculum vitae* was enclosed with a previous Rule 132 Declaration submitted on August 9, 2005.

2. **Exhibit A** depicts the results of experiments carried out by me or under my direction. In these experiments, human peripheral blood leukocytes (PBLs) were purified from three HLA-matched healthy donors. From these purified PBLs, about 70-85% pure human monocytes and about 80-90% pure human T-cells were separately generated using negative selection with various antibodies. The purified monocytes were then used to generate immature dendritic cells (DCs) by culturing in cytokines (*e.g.*, GM-CSF and IL-4). Immature dendritic cells were then loaded with two different antigen-antibody complexes, namely the B43.13 / CA 125 complex and the AR9.6 / CA 125 complex. AR9.6, like B43.13, is a murine monoclonal IgG1 antibody against CA 125; however, AR9.6 binds a different CA 125 epitope than B43.13. Immature DCs loaded with either of the two antigen-antibody complexes (or as controls, loaded with either CA 125 alone, B43.13 alone, AR9.6 alone, or medium) were further matured with TNF-α and IFN-α, and were used to stimulate the purified T-cells. T-cell activation was subsequently assessed using two independent assays, the Intracellular Cytokine (ICC) staining for IFN-γ, or the Cytotoxic T Lymphocyte (CTL) Assay for killing CA 125-positive ovarian cancer cells NIH:OVCAR-3 (ATCC).

3. The first and the second graphs in **Exhibit A** show that, based on the CTL assay, both the B43.13 / CA 125 complex, and the AR9.6 / CA 125 complex significantly stimulated T-cell activation after 3 or 4 rounds of stimulation compared to either antibody alone, antigen alone, or the negative control (media alone).

4. The third, fourth, and fifth graphs in **Exhibit A** show that, based on the ICC assay, both the B43.13 / CA 125 complex, and the AR9.6 / CA 125 complex significantly stimulated T-cell activation in all three donors after 4 rounds of stimulation compared to either antibody alone, antigen alone, or the negative control (media alone).

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code and that willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

Dated: Feb. 8, 2007 Signature: _____
Birgit C. Schultes, Ph.D.

Exhibit A

1. Materials and Methods

1.1. *Human Peripheral Blood Leukocytes*

Leukaphoresis samples from healthy normal donors expressing HLA-A2 were obtained from SeraCare LifeSciences (Oceanview, CA). Peripheral Blood Leukocytes (PBL) were purified on Ficoll (Histopaque 1.077, Sigma, St. Louis, MO), aliquoted and frozen.

1.2. *Antibodies*

MAb-B43.13 and AR9.6 are murine monoclonal IgG1 antibodies to CA125 (Unither Pharmaceuticals.).

1.3. *Antigens*

CA125 was purified from tissue culture supernatant of NIH:OVCAR-3 cells purchased from ATCC (Manassas, VA).[i] The purification steps included ammonium sulfate cut, diafiltration, gel filtration and an anti-CA125 antibody affinity column.

1.4. *Monocyte, Dendritic Cell (DC) and T Cell Preparation*

Human monocytes were prepared from PBL's after negative selection with anti-CD2, CD7, CD16, CD19, and CD56 antibodies, followed by anti-mouse-Ig-magnetic beads (Dynal-Invitrogen Monocyte Negative Selection Kit). These negatively selected cells were 70-85% pure monocytes as characterized using a broad CD marker panel and forward and side scatter by flow cytometry (BD FACSCalibur™ flow cytometer and BD CellQuest™ software). To generate immature DCs, monocytes were cultured in GM-CSF and IL-4 (1000 U/mL each, R&D Systems, Minneapolis, MN) for 6 days in RPMI + 2% human AB serum.

To induce DC maturation, Tumor Necrosis Factor (TNF)-α (10 ng/mL, Biosource) and Interferon (IFN)-α (50 U/mL, PBL Biomedical Laboratories) were added to immature DCs on Day 6 of culture. Mature DCs were characterized using a broad CD marker panel and gating based on forward and side scatter by flow cytometry on Day 7.

T cells were prepared from PBL by negative selection with anti-CD14, CD16, CD56, and HLA-DR/DP, followed by anti-mouse-Ig-magnetic beads (Dynal T Cell Negative Selection Kit). These negatively selected cells were approximately 80-90% pure T cells as characterized using a T cell and lineage CD marker panel as well as gating based on forward and side scatter by flow cytometry. T cells were used immediately following isolation.

1.5. *T Cell Activation Assays with DC*

Immature DCs were loaded with antigen, antibody or antigen-antibody complexes at Day 6 and matured with TNF-α and IFN-α (10 ng/mL and 50 U/mL, respectively) 2-4

Exhibit A

h later. On Day 7, DC were washed and purified T cells were added at a ratio of 10:1 (T cells to DC). Two or three stimulation rounds were performed. Before analyzing the T cells for activation, T cells were re-stimulated with antigen loaded DC for 24 h at a T cell:DC ratio of 20:1.

1.6. Quantitation of T Cell Activation by Intracellular Cytokine (ICC) Staining for IFN-γ

Cells were incubated with Golgi-Plug (R&D Systems) 2 h after stimulation with the antigen-loaded DC, incubated for another 16-20 h, blocked and stained with anti-CD3-FITC and anti-CD8-Cy-Chrome (all from Pharmingen) for 30 min on ice. Cells were washed, permeabilized with Perm/Fix (Pharmingen) for 30 min on ice, washed and stained with anti-IFN-γ-PE antibody (Pharmingen) for 30 min on ice. Cells were washed, fixed in 0.5% formalin and analyzed by flow cytometry (FACS Calibur, Becton Dickinson), gating on CD3-positive lymphocytes.

1.7. Cytotoxic T Lymphocyte Assay (CTL)

T cells were harvested 24 h after stimulation with loaded DC and used to set-up the CTL assay. As targets cells, CA125-positive HLA-A2 expressing ovarian cancer cells (NIH:OVCAR-3, ATCC) were used. The target tumor cells (1-2x10^6 cells) were labeled with Calcein-AM (Molecular Probes) according to the manufacturer's instructions. The cells were incubated for 30 min. at 37°C with occasional mixing followed by washing the cells in 3-times with 10 mL of medium. The cells were resuspended at 1x10^5 cells/mL, and 100 µL was distributed into round-bottom microtiter plates.

T cells from each group were washed and resuspended at 1, 0.3 and 0.1 x10^6 cells/mL, and 100 µL/well for each dilution were plated to the labeled target cells in triplicates, including controls with target cells alone (spontaneous release) and target cells lysed with 0.1% Triton X-100 (maximum release). The plates were incubated for 4 h at 37°C after a 3 min centrifugation at 30 x *g*. The Calcein-AM release (tumor killing) into the supernatant was measured with a fluorescence plate reader.

1.8. Analysis of ICC and CTL

For the ICC experiments, the % positive cell data were acquired on the flow cytometer and gates set based on isotype controls. For the CTL assay the specific lysis was calculated based on the following formula:

$$\% \text{ lysis} = \frac{\text{mean released test results} - \text{mean spontaneous released results}}{\text{mean maximum released results} - \text{mean spontaneous released results}}$$

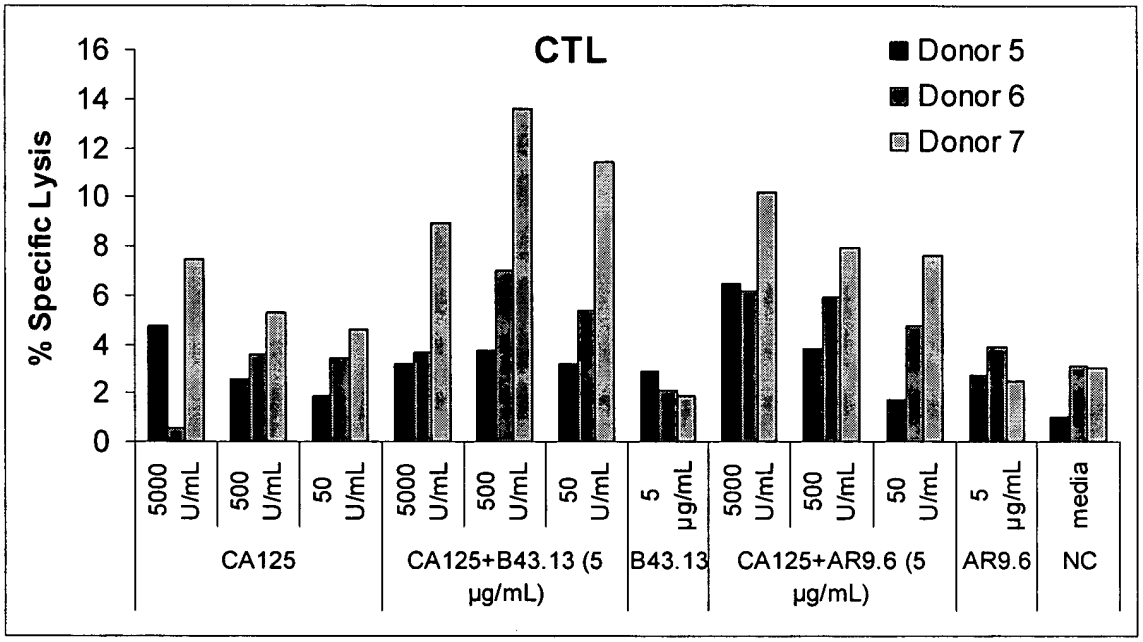
Exhibit A

2. Results

2.1. *CTL Assay after 3 Rounds of Stimulation*

T cells were analyzed 24 h after the 3rd stimulation with loaded DC for lysis of CA125-positive tumor cells using three different donors. Lysis for T cells stimulated with CA125 alone (50-5000 U/mL) or with antibody alone (5 μg/mL) ranged form 2-7.5%. Immune complexes of CA125 and B43.13 or AR9.6 at the same concentrations stimulated T cells more effectively, resulting in lysis of up to 14% of the tumor cells.

GRAPH 1



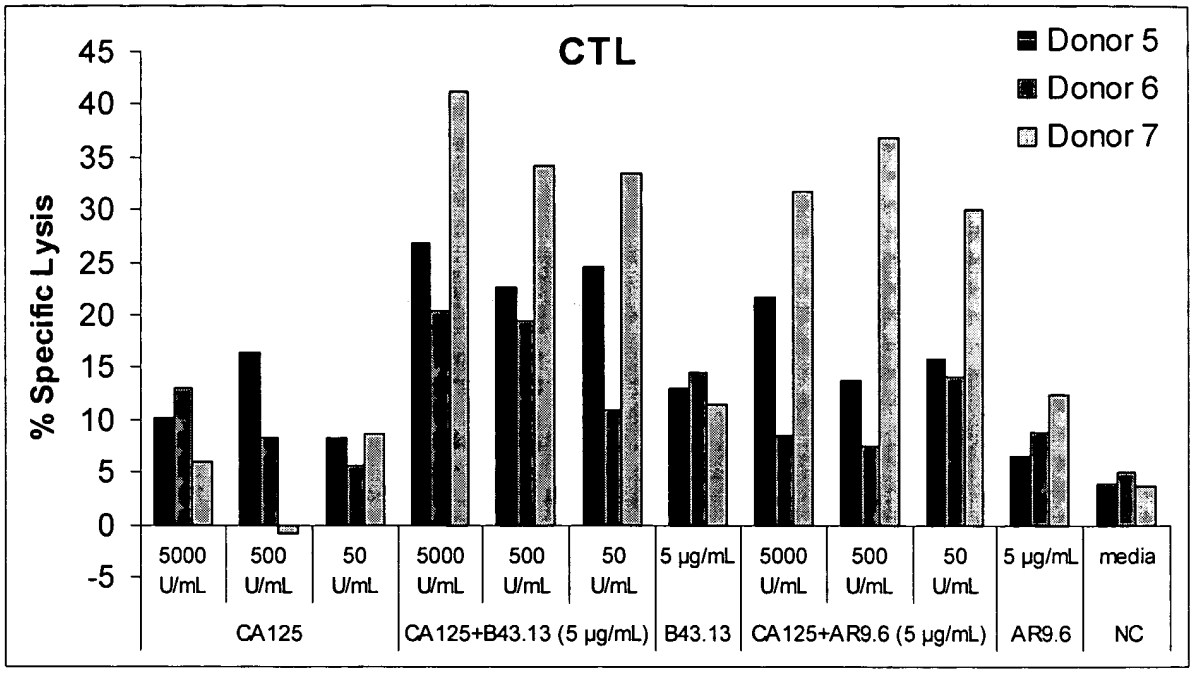
Immature DC from 3 donors were loaded with CA125 alone (50, 500, and 5,000 U/mL), MAb-B43.13 or AR9.6 (5 μg/mL), CA125 + MAb-B43.13 (50, 500, or 5,000 U/mL + 5 μg/mL), or CA125 + AR9.6 (50, 500, or 5,000 U/mL + 5 μg/mL). DC were matured for 24 hrs and then washed 2 times with media. Purified T cells from the matching donor were added and incubated for 7 days. T cells were re-stimulated with DC loaded as before for an additional 2 rounds. Twenty-four hours after the final stimulation, T cells were harvested and analyzed for lysis of NIH:OVCAR 3 cells in a Calcein-AM release assay.

2.2. *CTL Assay after 4 Rounds of Stimulation*

T cells were analyzed 24 hrs after the 4th stimulation with loaded DC for lysis of CA125-positive tumor cells using three different donors. Lysis for T cells stimulated with CA125 alone or with antibody alone ranged form 0-17%. Processing of immune complexes of CA125 and B43.13 or AR9.6 by autogous DC resulted in significantly higher CTL activation. Tumor cell lysis ranged from 12-41%.

Exhibit A

GRAPH 2

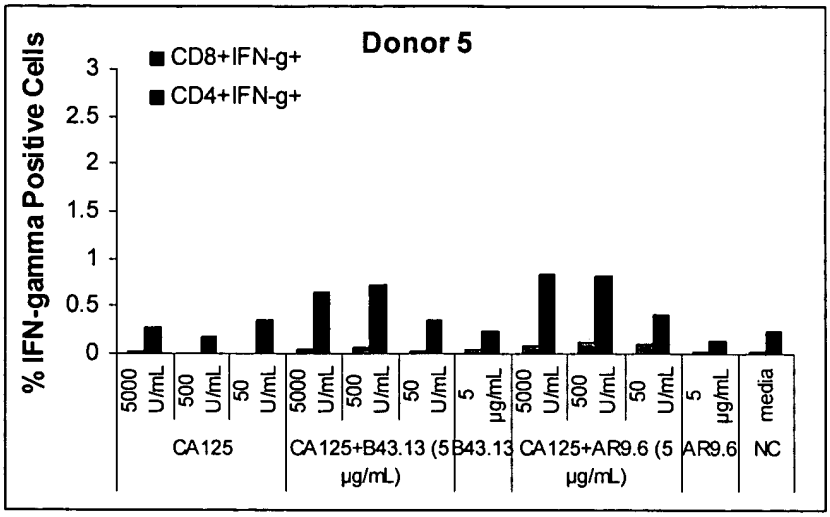


Immature DC from 3 donors were loaded with CA125 alone (50, 500, and 5,000 U/mL), MAb-B43.13 or AR9.6 (5 µg/mL), CA125 + MAb-B43.13 (50, 500, or 5,000 U/mL + 5 µg/mL), or CA125 + AR9.6 (50, 500, or 5,000 U/mL + 5 µg/mL). DC were matured for 24 hrs and then washed 2 times with media. Purified T cells from the matching donor were added and incubated for 7 days. T cells were re-stimulated with DC loaded as before for an additional 3 rounds. Twenty-four hours after the final stimulation, T cells were harvested and analyzed for lysis of NIH:OVCAR 3 cells in a Calcein-AM release assay.

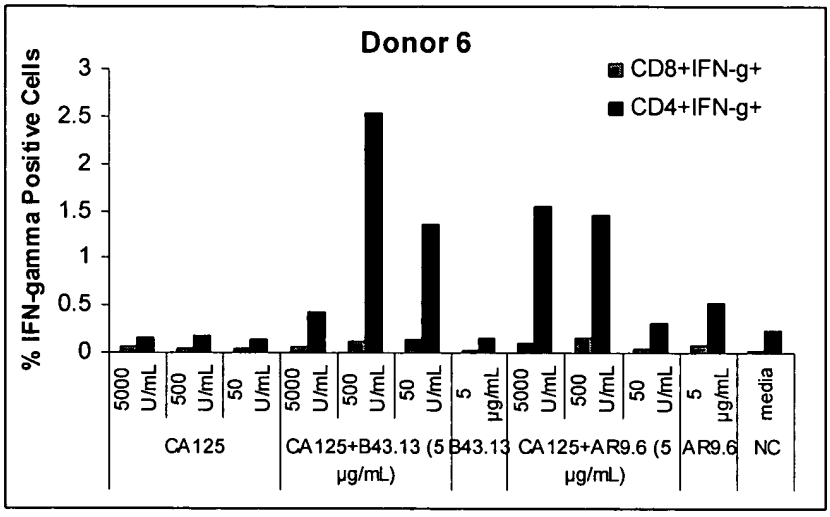
2.3. *Intracellular IFN-γ Staining after 4 Rounds of Stimulation*

T cells were stimulated with DC loaded with CA125 alone, B43.13 alone, AR9.6 alone, or immune complexes of CA125+B43.13 or CA125+AR9.6 for 4 rounds. Two hours after the final stimulation, wells were treated with Brefeldin A to block cytokine secretion and incubated overnight. The following day, T cells were harvested and washed, stained for surface CD3 and CD8, fixed, permeabilized and stained for intracellular IFN-γ. Cells were analyzed by flow cytometry. Results from 3 different donors are shown. T cell activation for cells stimulated with DC processing CA125 alone or antibody alone was significantly lower than for cells stimulated with DC that processed immune complexes of CA125+B43.13 or CA125+AR9.6.

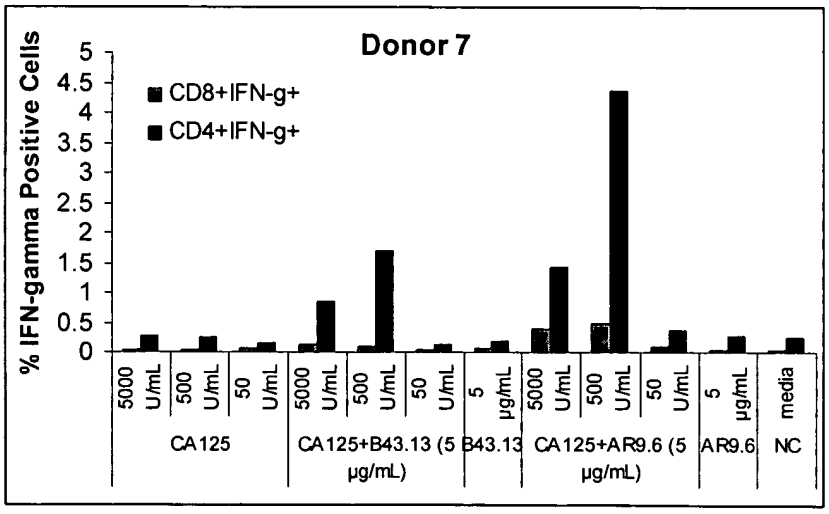
Exhibit A



GRAPH 3



GRAPH 4



GRAPH 5

Exhibit A

Immature DC from 3 donors were loaded with CA125 alone (50, 500, and 5,000 U/mL), MAb-B43.13 or AR9.6 (5 μg/mL), CA125 + MAb-B43.13 (50, 500, or 5,000 U/mL + 5 μg/mL), or CA125 + AR9.6 (50, 500, or 5,000 U/mL + 5 μg/mL). DC were matured for 24 hrs, and then washed 2 times with media. Purified T cells from the matching donor were added and incubated for 7 days. T cells were re-stimulated with DC loaded as before for an additional 3 rounds. Wells were treated with Brefeldin A and 24 hrs after the final stimulation, T cells were harvested, stained for CD3, CD8 and IFN-γ and analyzed by flow cytometry.

3. References

[i] Schultes BC, Baum RP, Niesen A, Noujaim AA, Madiyalakan R. Anti-idiotype induction therapy: anti-CA125 antibodies (Ab_3) mediated tumor killing in patients treated with Ovarex mAb (Ab_1). Cancer Immunol and Immunther (1998) 46:201-212.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

re Patent Application of:
Madiyalakan *et al.*

Confirmation No.: 4505

Application No.: 09/152,698

Art Unit: 1643

Filed: September 2, 1998

For: THERAPEUTIC COMPOSITIONS THAT PRODUCE AN IMMUNE RESPONSE

Examiner: K. A. Canella

VERIFIED STATEMENT UNDER 37 C.F.R. § 1.804(b)
REGARDING BIOLOGICAL DEPOSIT

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Birgit C. Schultes, an inventor of the instant application, hereby provide the following verified statement regarding certain biological material deposits made with an International Depository authority - the American Type Culture Collection (ATCC) - under the provisions of the Budapest Treaty:

1. The mouse hybridoma B43.13 (MCB-ALT-96), which produces the antibody B43.13, was deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209, on May 18, 2000, and was given ATCC deposit number PTA-1883.

2. I have reviewed the specification of U.S.S.N. 09/152,698, filed on September 2, 1998.

3. To the best of my knowledge, the above-referenced deposited hybridoma is the biological material producing the monoclonal antibody as described in the specification as filed of U.S.S.N. 09/152,698.

Dated: Feb. 1, 2007 Signature: B. Schultes
Birgit C. Schultes

ATCC

10801 University Blvd • Manassas, VA 20110-2209 • Telephone: 703-365-2700 • FAX: 703-365-2745

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Altarex Corporation
Attn: Birgit Schultes
303 Wyman Street
Suite 125
Waltham, MA 02451

Deposited on Behalf of: Altarex Corporation

Identification Reference by Depositor:	**Patent Deposit Designation**
Mouse-mouse hybridoma B43.13: MCB-ALT1-96	PTA-1883

The deposit was accompanied by: __ a scientific description a proposed taxonomic description indicated above.

The deposit was received May 18, 2000 by this International Depository Authority and has been accepted.

AT YOUR REQUEST: X We will inform you of requests for the strain for 30 years.

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strain.

If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with living culture of the same.

The strain will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested August 2, 2000. On that date, the culture was viable.

International Depository Authority: American Type Culture Collection, Manassas, VA 20110-2209 USA.

Signature of person having authority to represent ATCC:

Barbara E. Coupé

Barbara E. Coupé, Administrator, Patent Depository

Date: August 3, 2000

cc: Wayne Keown (Ref: Docket or Case No.: A52024 US, A52021 US, A52023.2 US, A52023.1 US)

BP4/9

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